Sirtuin 5 as a novel target to blunt blood-brain barrier damage induced by cerebral ischemia/reperfusion injury

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Abstract: BACKGROUND: In acute ischemic stroke (AIS) patients, impaired blood-brain barrier (BBB) integrity is associated with hemorrhagic transformation and worsened outcome. Yet, the mechanisms underlying these relationships are poorly understood and consequently therapeutic strategies are lacking. This study sought to determine whether SIRT5 contributes to BBB damage following I/R brain injury. METHODS AND RESULTS: SIRT5 knockout (SIRT5-/-) and wild type (WT) mice underwent transient middle cerebral artery (MCA) occlusion (tMCAO) followed by 48h of reperfusion. Genetic deletion of SIRT5 decreased infarct size, improved neurological function and blunted systemic inflammation following stroke. Similar effects were also achieved by in vivo SIRT5 silencing. Immunohistochemical analysis revealed decreased BBB leakage and degradation of the tight junction protein occludin in SIRT5-/- mice exposed to tMCAO as compared to WT. In primary human brain microvascular endothelial cells (HBMVECs) exposed to hypoxia/reoxygenation (H/R), SIRT5 silencing decreased endothelial permeability and upregulated occludin and claudin-5; this effect was prevented by the PI3K inhibitor wortmannin. Lastly, SIRT5 gene expression was increased in peripheral blood monocytes (PBMCs) of AIS patients at 6h after onset of stroke compared to sex- and age-matched healthy controls. CONCLUSION: SIRT5 is upregulated in PBMCs of AIS patients and in the MCA of WT mice exposed to tMCAO; SIRT5 mediates I/R-induced brain damage by increasing BBB permeability through degradation of occludin. This effect was reproduced in HBMVECs exposed to H/R, mediated by the PI3K/Akt pathway. Our findings shed new light on the mechanisms of I/R-dependent brain damage and suggest SIRT5 as a novel therapeutic target.

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Sirtuin 5 Mediated-Occludin Degradation Contributes to Blood-Brain Barrier Breakdown following Ischemia-Reperfusion Brain Injury in Mice

Diaz-Cañestro, Sirtuin 5 contributes to BBB breakdown after stroke

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Abstract

Background and Purpose- Acute ischemic stroke (AIS) is intrinsically associated with age and its incidence is expected to increase along with the growing life expectancy. In AIS patients, impaired blood brain barrier (BBB) integrity is linked with intracerebral hemorrhage and worsened outcome. However, the mechanisms underlying this link are poorly understood thus specific therapeutic strategies to address it are lacking. Sirtuin 5 (SIRT5) is a member of the NAD (+)-dependent protein deacetylase family previously shown to be protective in cardiac ischemia/ reperfusion (I/R) injury. Yet, little is known about its putative role in I/R-induced brain injury. The present study investigates the role of SIRT5 in a mouse model of I/R brain injury induced by transient middle cerebral artery occlusion (tMCAO) and in primary human brain microvascular endothelial cells (HBMVECs) exposed to hypoxia/reoxygenation (H/R).

Methods and results- SIRT5 knockout ($SIRT5^{-/-}$) and wild type (WT) mice were subjected to 45 min of ischemia followed by 48 h of reperfusion. Deletion of SIRT5 decreased infarct size, improved neurological function and blunted systemic inflammation following stroke. Similarly, SIRT5 knockdown by siRNA decreased infarct size as compared to scrambled treated mice. Immunohistochemical analysis revealed decreased BBB leakage and occludin- a tight junction protein (TJP) - degradation in $SIRT5^{-/-}$ mice exposed to tMCAO as opposed to WT. In primary HBMVECs exposed to H/R, knockdown of SIRT5 decreased endothelial permeability and upregulated occludin and claudin-5 protein levels; this effect was prevented by pretreatment of HBMVECs with the PI3K inhibitor wortmannin.

Conclusion- SIRT5 mediates I/R-mediated brain damage by increasing BBB permeability through degradation of the TJP occludin. This effect was also reproduced in HBMVECs exposed to H/R and was mediated by the PI3K/ Akt pathway thus, underlying the translational relevance of our findings. Our data shed new light on the mechanisms of I/R-dependent brain damage and indicate SIRT5 as an interesting novel therapeutic target.

Key words: SIRT5, blood brain barrier, tight junction proteins, PI3K/ Akt pathway

Introduction

Stroke is the second-leading cause of death and a major cause of disability worldwide and its incidence is set to increase due to the currently growing life expectancy.[1-3] In absolute numbers, 15 million people suffer stroke annually and, from these, more than 10 million die or are permanently disabled. Four out of five strokes are categorized as acute ischemic stroke (AIS), for which the only currently available treatments are thrombolytic agent and/or use of a mechanical clot retrieval device.[4] However, unlike the success of reperfusion therapy in myocardial infarction, its usage in AIS patients is limited to less than 10% of all cases with no alternative treatment.[5]

Accumulating evidence indicates the blood brain barrier (BBB) as a promising therapeutic target for AIS. The BBB is formed by a monolayer of microvascular endothelial cells joined together by tight junction proteins (TJPs), restricting the passage of substances into the central nervous system (CNS). During ischemia and subsequent reperfusion, the integrity of the BBB is disrupted resulting in the leakage of blood-borne proteins in the parenchyma. In clinical studies, BBB leakage is strongly associated with hemorrhagic transformation and worsened outcome in AIS patients.[6, 7] As for the mechanisms, the vast majority of in vivo and in vitro studies coincide in that TJPs disruption and matrix metalloproteinase (MMP)-dependent extracellular matrix degradation are the two crucial events accounting for BBB breakdown after stroke.[8-11] In this regard, oxidative stress and inflammatory response are thought to be fundamental mediators.[12] Yet, the molecular pathways involved in BBB disruption remain to be elucidated.

Mammalian sirtuins (SIRTs) are a family of seven proteins associated with distinct biological functions such as control of aging, oxidative stress, inflammation, apoptosis and metabolic pathways. SIRTs mediate their effects by means of post-translational protein modifications. In particular, sirtuin 5 (SIRT5) is a NAD+-dependent protein desuccinylase and demalonylase, as well as a weak deacetylase. Multiple (patho-) physiological functions of SIRT5 are currently emerging. Of interest, SIRT5 knockout mice exhibit a 75 % increment in infarct size

following myocardial ischemia/reperfusion (I/R) injury.[13] However, whether SIRT5 also plays any role in I/R brain injury remains unknown.

In the present study, we investigated the effects of deletion and knockdown of SIRT5 on I/R-induced brain damage with a specific focus on BBB integrity and TJPs expression using both *in vivo* transient middle cerebral artery occlusion (tMCAO) and *in vitro* hypoxia-reoxygenation (H/R) stroke models.

Methods

Animals

Experiments were performed on 10–14-week-old male SIRT5 knockout ($SIRT5^{-/-}$) mice and wild-type (WT) controls (C57BL/6J). $SIRT5^{-/-}$ mice were generated as described previously.[14] Briefly, $SIRT5^{floxed}$ mice were obtained by standard gene targeting procedures,[15] using 129SV embryonic stem (ES) cells. Then, these animals were crossed with CMV-Cre transgenic mice[16] to generate germline $SIRT5^{-/-}$ mice, which have been subsequently backcrossed for 5 generations with C57BL/6J mice.

In vivo SIRT5 silencing was performed as previously described.[17] Briefly, pre-designed small interfering RNA (siRNA) targeting SIRT5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were injected intravenously in a randomized way. As a negative control, scrambled siRNA (Microsynth, Balgach, Switzerland) was used (sense: 5'-UACACACUCUCGUCUCU[dT][dT]-3'; antisense: 5'-AGAGACGAGAGUGUGUA[dT][dT]-3'). 24 h before the surgical procedure, 1.6 nmol of siRNA were incubated with a mixture of 150 mM NaCl solution–jetPEI® (Polyplus TransfectionTM, New York, NY, USA) delivery reagent for 15 min and injected into the tail vein.

Animals were maintained at 24ºC under a 12 h light/dark cycle and were fed on a normal chow diet with ad libitum access to food and water. Study design and experimental protocols were approved by the institutional animal care committee (License no. TVA 293_2014; Kommission für Tierversuche des Kantons Zürich, Switzerland).

Middle Cerebral Artery Occlusion

tMCAO was performed to induce I/R brain injury, as described previously.[18, 19] Mice were anaesthetized using isoflurane 3 % and 1.5 % for induction and maintenance respectively, while body temperature was kept at 37°C. For analgesia, 0.5% bupivacaine was infiltrated at the incision side. The common, internal and external carotid arteries were dissected and a 6-0 silicone-coated filament (Doccol Corporation, Sharon, MA, USA) was inserted into the common carotid artery and advanced to the origin of the left MCA to prompt tMCAO for 45 min. After ischemia, middle cerebral artery (MCA) reperfusion was allowed for 24 h or 48 h. During anesthesia, regional cerebral blood flow (rCBF) in the area of the cortex supplied by the MCA was measured using laser Doppler flowmetry (PeriFluxSystem 5000 with probe model no. 418-1, Perimed AB, Järfälla, Sweden). The microtip probe was positioned and glued ~2 mm posterior and 6 mm lateral to the bregma.

Stroke Volume

Murine brains were cut into 4 equally spaced (2 mm) coronal sections and immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, Chemie GmbH, Buchs, Switzerland) at 37°C for 20 min. Areas of infarction, ipsilateral and contralateral hemispheres were quantified using ImageJ software (Image J, NIH, MD, USA). To correct infarct size measurement for cerebral swelling and subsequent overestimation, we applied the following formula as already described:[20] corrected infarct volume = contralateral hemisphere volume - (ipsilateral hemisphere volume - infarct volume). The infarct size was expressed as a percentage of the total contralateral hemisphere volume.

Neurological Deficiency Assessment

Neurological status was assessed by a four-point scale neurological score test according to Bederson et al.[21]: grade 0, normal neurological function; grade 1, forelimb flexion; grade 2, circling; grade 3, leaning to the contralateral side at rest; grade 4, no spontaneous motor

activity. An experimentator blinded to the group allocation evaluated the neurological deficit at 2 h, 24 h and 48 h after tMCAO.

Blood Sampling and Cell Count

Blood samples were taken 48 h after tMCAO for measuring leukocytes, red blood cells and platelet counts using an impedance hematology analyzer (ScilVet ABCplus; Horiba, Kyoto, Japan). Data were expressed as neutrophils and lymphocytes percentages (relative to the total white blood cells) and neutrophil to lymphocytes ratio.

Histology

Histological procedures were performed as previously described.[22] Briefly, following induction of deep anesthesia, mice were transcardially perfused with phosphate buffered saline (PBS) and 4.0% paraformaldehyde (PFA) (Sigma-Aldrich, Chemie GmbH, Buchs, Switzerland) in PBS at room temperature, followed by incubation in 30% sucrose in PBS for 36 h. Cryoprotected brains were cut into 100-µm thick free-floating sections pre-treated with proteinase K for antigen retrieval and immune-blocked. After these, they were incubated with primary antibodies at the following dilution occludin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), the endothelial marker CD31 (1:50; BD Pharmingen, Allschwil, Switzerland) and claudin-5 (1:200; Abcam, Cambridge, United Kingdom) at 4ºC overnight, respectively. After washing, brain sections were incubated with the appropriate secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) for 24 h at room temperature. Images were acquired using a confocal microscope (Leica SP8; Leica, Wetzlar, Germany). Stained area of claudin-5 and occludin was measured using ImageJ Software (Image J, NIH, MD, USA) and normalized to the total endothelial cell surface assessed by CD31 staining.

BBB permeability was measured assessing endogenous immunoglobulin G (IgG) extravasation, sections were blocked for 60 min followed by incubation with Alexa647-conjugated donkey anti-mouse IgG for 24 h (1:600; Jackson Immunoresearch, West Grove,

USA). IgG leakage was corrected for edema, as previously described in stroke volume assessment, and expressed as a percentage of the total contralateral hemisphere volume.

Cell Culture Experiments

Primary human brain microvascular endothelial cells (HBMVECs) (Cell Systems) between passage 6 and 8 were used for *in vitro* experiments. Endothelial cells were cultured in EBM-2 medium (Lonza, Bettlach, Switzerland), supplemented with EGM-2 bullet kit (Lonza) and 10% fetal bovine serum. Cells were grown to 80 % confluence before being transfected with SIRT5 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or scrambled siRNA (Microsynth, Balgach, Switzerland) for 24 h using Lipofectamine 3000 transfection kit according to manufacturer's recommendation (Invitrogen, Carlsbad, CA, USA). Next, cells were exposed to hypoxia (0.2% oxygen) for 4 h followed by 4 h of reoxygenation (21% oxygen) or kept at normoxic condition (21% oxygen) for 8 h. Wortmannin (Enzo Life Sciences, Lausen, Switzerland) a PI3K inhibitor, in a final concentration of 10^{-7} M or dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Chemie GmbH, Buchs, Switzerland) vehicle treatment was added to the HBMVECs cells 5 min before to hypoxia. Hypoxia was induced using a gas-controlled glove hypoxia workstation (Invivo$_2$ 400, Baker Ruskinn, Sanford, ME, USA).

Measurement of Barrier Function by Transendothelial Electrical Resistance

Measurements of transendothelial electrical resistance (TEER) on HBMVEC monolayers were performed using the electric cell-substrate impedance system (ECIS) Z Theta system (Applied Biophysics, Troy, NY, USA) as previously described.[23] The ECIS system provides real-time monitoring of changes in TEER. In brief, HBMVECs at $9x10^5$/well were plated on fibronectin coated 8W10E+ electrode arrays (Applied Biophysics). Then, cells were allowed to form monolayers until stable TEER values were reached. After 5 h, the monolayers were treated with SIRT5 or scrambled siRNA as previously specified. Then, they were exposed to hypoxic conditions for 4 h followed by reoxygenation for 48 h. Wortmannin (10^{-7} M) or vehicle treatment (DMSO) was applied 5 min before to hypoxia. Measurements were conducted at

multiple frequencies ranging from 62.6 Hz to 64 kHz. Data were expressed as TEER percent change from baseline values (before hypoxia).

Protein expression studies

Protein expression was determined by Western blot analysis. Endothelial cells and aortae were homogenized in lysis buffer (Tris 50 mM, NaCl 150 mM, EDTA 1 mM, NaF 1 mM, DTT 1 mM, aprotinin 10 mg/mL, leupeptin 10 mg/mL, Na_3VO_4 0.1 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, and NP-40 0.5%). Protein concentration was determined according to the manufacturer's recommendations (Bio-Rad Laboratores AG, Fribourg, Switzerland); 20 – 30 µg of total protein lysates were separated on an 8 or 10% SDS–PAGE before being transferred to a polyvinylidene fluoride membrane by wet transfer (Bio-Rad). Membranes were incubated with primary antibodies against SIRT5 (1:1000, Cell Signaling, Beverly, MA, USA), occludin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), claudin-5 (1:1000, Invitrogen, Carlsbad, CA, USA), phospho-Akt (1:1000, Cell Signaling, Beverly, MA, USA), Akt (1:1000, Cell Signaling, Beverly, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:40000, Merck Millipore, Billerica, MA,USA) at 4°C overnight on a shaker. Secondary antibodies (Southern Biotechnology, Birmingham, AL, USA) were applied for 1 h at room temperature. Densitometric analyses were performed (Amersham Imager 600, GE Healthcare Europe GmbH, Glattbrugg, Switzerland) and protein expression was normalized to GAPDH.

Coimmunoprecipitation

HBMVECs were lysed in ice-cold lysis buffer containing Tris 50 mM, NaCl 150 mM, EDTA 1 mM, NaF 1 mM, DTT 1 mM, aprotinin 10 mg/mL, leupeptin 10 mg/mL, Na_3VO_4 0.1 mM, PMSF 1 mM, and NP-40 0.5%. Lysates were centrifuged at 10,000 g to remove insoluble material. For immunoprecipitation, precleared lysates were incubated with Akt antibody overnight at 4°C. Lysates were precleared by incubation with 50 µL of protein A/G-agarose

for 4 h at 4°C with rocking. Agarose beads were pelleted by centrifugation at 1000 g. Immunoprecipitated proteins were eluted from the beads by boiling for 5 min in SDS sample buffer and immunoblotted with primary antibodies against Akt (1:1000), claudin-5 (1:1000) and occludin (1:1000). Bound antibody was visualized using an enhanced chemiluminescence system (Merck Millipore, Billerica, MA, USA) after incubation of the blot with peroxidase-conjugated secondary antibody for 1 h.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc, La Jolla, CA, USA). Results were confirmed to follow a normal distribution with the Kolmogorov-Smirnov test of normality. Data that passed the normality assumption were analyzed with two-tailed unpaired Student's t-test and data that failed the normality assumption were analyzed with the nonparametric Mann–Whitney *U* test. For repeated measurements, two-way ANOVA with Sidak *post hoc* test was applied. A probability value (P) below 0.05 was considered statistically significant.

Results

Deletion as well as knockdown of SIRT5 reduces infarct size after ischemia/reperfusion brain injury

To investigate the role of SIRT5 in stroke, $SIRT5^{-/-}$ and WT mice underwent MCAO for 45 min followed by 48 h of reperfusion. Measurement of rCBF by laser Doppler flowmetry in the region perfused by the MCA was similar between genotypes (*P* = NS for all time points; n = 4-6; **Figure S_1 in Supplementary material**). Deletion of SIRT5 reduced infarct size assessed by TTC staining at 48 h after tMCAO (WT: 40.93 ± 3.32% vs. $SIRT5^{-/-}$: 19.60 ± 3.67%; *P* = 0.0005; n = 9; **Figure 1A-B**). Reduced infarct size in $SIRT5^{-/-}$ mice was paralleled by reduced neurological deficit at 48 h compared to WT mice (WT: 1.07 ± 0.86 vs. $SIRT5^{-/-}$: 0.41 ± 0.66; *P* = 0.03; n = 13-12; **Figure 1C**).

To confirm these results using a different approach, *in vivo* knockdown of SIRT5 was performed by intravenous (i.v.) administration of SIRT5 siRNA mixed with a cationic transfection reagent. First, SIRT5 silencing efficiency was assessed *in vivo* by measuring SIRT5 protein levels in aortic arteries. SIRT5 siRNA treated mice (siSIRT5) exhibited a reduced SIRT5 protein expression compared to scrambled siRNA treated mice (siSCR) at 48 h after siRNA injection (siSCR: 1.00 ± 0.08 vs. siSIRT5: 0.61 ± 0.16; P = 0.046; n = 8-6; **Figure S_2A in the Supplementary material**). In line with the SIRT5$^{-/-}$ mice data, siSIRT5 mice presented reduced stroke size compared to siSCR mice at 24 h after tMCAO (siSCR: 33.07 ± 1.83% vs. siSIRT5: 21.83 ± 3.05%; P = 0.004; n = 10-7; **Figure 1D-E**). There was not difference between groups in terms of post-stroke neurological score (data not shown).

Deletion of SIRT5 reduces BBB disruption and tight junction protein (TJP) degradation

I/R-induced BBB breakdown is characterized by extravasation of large circulating molecules, such as IgG into the affected parenchyma.[24] As expected, IgG leakage was observed in the ipsilateral hemisphere of both WT and SIRT5$^{-/-}$ mice after stroke, while no IgG staining was found in the contralateral hemisphere (**Figure 2A**). IgG extravasation was significantly reduced in the SIRT5$^{-/-}$ mice compared to WT 48 h after tMCAO in the ipsilateral hemisphere (WT: 72.12 ± 4.37% vs. SIRT5$^{-/-}$: 44.74 ± 7.77 %; P = 0.0106; n = 6-5; **Figure 2B**).

BBB permeability is regulated by tight and adherens junctional proteins. During stroke the paracellular permeability increases due to the degradation of these proteins. Herein, we assessed the integrity of two transmembrane TJPs, occludin and claudin-5, using immunostainings. A reduction in occludin levels in the ipsilateral hemisphere of WT and SIRT5$^{-/-}$ mice compared to the contralateral hemisphere was observed (WT contralateral: 0.82 ± 0.06 vs. WT ipsilateral: 0.43 ± 0.04; P = 0.0005; n=6; SIRT5$^{-/-}$ contralateral: 0.86 ± 0.06 vs. SIRT5$^{-/-}$ ipsilateral: 0.61 ± 0.06; P = 0.025; n = 5; **Figure 2C-D).** However, occludin levels were higher in the ipsilateral hemisphere of SIRT5$^{-/-}$ mice compared to WT mice (WT: 0.43 ± 0.04 vs. SIRT5$^{-/-}$: 0.61 ± 0.064; P=0.040; n=6-5; **Figure 2C-D**). Similarly, claudin-5 was decreased in the ipsilateral hemisphere of both groups compared to the contralateral

hemisphere (WT contralateral: 0.59 ± 0.10 vs. WT ipsilateral: 0.11 ± 0.24; *P* = 0.002; n=6; SIRT5$^{-/-}$ contralateral: 0.48 ± 0.05 vs. SIRT5$^{-/-}$ ipsilateral: 0.21 ± 0.06; *P* = 0.01; n = 5; **Figure 2E-F**). The expression of claudin-5 did not differ between groups in the ipsilateral hemisphere (WT: 0.11 ± 0.02 vs. SIRT5$^{-/-}$: 0.21 ± 0.065; *P*=0.18; n=5; **Figure 2E-F**).

Deletion of SIRT5 decreases the systemic inflammatory response

In ischemic stroke patients the number of circulating neutrophils increases few hours after the stroke onset while lymphocytes follow an opposite tendency.[25, 26] Thus, the neutrophil-to-lymphocyte ratio (NLR) is increased with stroke and correlates with stroke outcome and mortality.[27] We further investigated whether SIRT5 affects the systemic inflammatory response 48 h after stroke. Total white blood cells displayed not difference between the study groups (WT: 2.41 ± 0.58 10^9/L vs. SIRT5$^{-/-}$: 2.27 ± 0.49 10^9/L; *P* = 0.86; n = 7-5; **Figure 3A**). However, SIRT5$^{-/-}$ mice showed higher lymphocytes counts (WT: 48.75 ± 2.25% vs. SIRT5$^{-/-}$: 60.88 ± 2.89%; *P* = 0.007; n = 7-5; **Figure 3B)** and lower neutrophils (WT: 46.14 ± 3.27% vs. SIRT5$^{-/-}$: 35 ± 2.88 %; *P* = 0.036; n = 7-5 **Figure 3C)** leading to a reduced NLR compared to WT mice (WT: 1.21 ± 0.18 vs. SIRT5$^{-/-}$: 0.68 ± 0.06; *P* = 0.043; n = 7-5 **Figure 3D**).

Knockdown of SIRT5 regulates BBB permeability after H/R in HBMVECs

To investigate the mechanisms underlying the blunted BBB damage and to translate our *in vivo* murine data to human cells, we established an *in vitro* BBB model consisting of a monolayer of HBMVECs seeded at confluence in ECIS electrode chambers. Furthermore, to mimic ischemia and reperfusion occurring in the mouse, HBMVECs were subjected to hypoxia for 4 h followed by 48 h of reoxygenation and TEER values were measured during the entire experiment. SIRT5 siRNA treated cells (siSIRT5) showed higher resistance compared to scramble treated cells (siSCR) (**Figure 4B**). The difference between this two groups became evident 32 h after hypoxia and was augmented overtime confirming the anti-permeability properties of SIRT5 knockdown (32 h: siSCR: 0.43 ± 0.04 vs. siSIRT5: 0.59 ± 0.04; *P* = 0.01; n = 10; **Figure 4B**). We hypothesized that this effect was mediated by

PI3K/Akt pathway since p-Akt/Akt ratio was increased upon knockdown of SIRT5 (siSCR: 1.24 ± 0.10, siSCR H/R: 1.00 ± 0.16 vs. siSIRT5 H/R**:** 1.47 ± 0.11; *P* = 0.04; n = 6; **Figure 4A**). According to our hypothesis, the PI3K/Akt pathway inhibitor - wortmannin - blocked the effect of SIRT5 siRNA on TEER (32 h: siSIRT5: 0.59 ± 0.04 vs. siSIRT5 + WM**:** 0.45 ± 0.04; *P* = 0.04; n = 10; **Figure 4B**).

To further elucidate the potential mechanisms accounting for the effect of SIRT5 knockdown on BBB permeability, we assessed early responses on tight and adherens junctional proteins after H/R. After 4 h of hypoxia followed by 4 h of reoxygenation, claudin-5, occludin and VE-cadherin expression did not differ compared to normoxic controls (data not shown). Nevertheless, siSIRT5 treatment increased occludin and claudin-5, but not VE-cadherin (data not shown), protein expression after H/R compared to siSCR (occludin: siSCR H/R: 1 ± 0.12 vs. siSIRT5 H/R**:** 1.33 ± 0.11; *P* = 0.04; n = 5; **Figure 4C;** claudin-5**:** siSCR H/R: 1 ± 0.11 vs. siSIRT5 H/R**:** 1.64 ± 0.24; *P* = 0.02; n = 7 **Figure 4D**). In order to investigate whether the PI3K/Akt pathway also mediates the upregulation of TJPs in the absence of SIRT5, siSIRT5 HBMVECs were treated with wortmannin or vehicle (DMSO) before the hypoxic exposure. In line with the results from endothelial permeability experiments, wortmannin prevented the increase of occludin and claudin-5 after siSIRT5 (occludin: siSIRT5 H/R**:** 1.33 ± 0.11 vs. siSIRT5 + WM H/R**:** 0.87 ± 0.17; *P* = 0.04; n = 5; **Figure 4C;** claudin-5**:** siSIRT5 H/R**:** 1.64 ± 0.24 vs. siSIRT5 + WM H/R**:** 0.64 ± 0.15; *P* = 0.01; n = 7 **Figure 4D**). Finally, we confirmed the knockdown of SIRT5 in HBMVECs. SIRT5 siRNA induced around 50% reduction in SIRT5 protein expression compared to SCR siRNA (siSCR: 1.00 ± 0.10 vs. siSIRT5: 0.47 ± 0.07; *P* = 0.006; n = 4; Figure S_2B in the Supplementary material).

SIRT5 mediates the interaction of claudin-5 with Akt

To confirm the involvement of the PI3K/Akt pathway in the upregulation of TJPs following SIRT5 knockdown, we performed a co-immunoprecipitation experiment. Hereby, we found that claudin-5 and occludin co-immunoprecipitate with Akt. In addition, knockdown of SIRT5 enhanced the binding of claudin-5 to Akt by approximately fourfold above the scramble-

treated group (**Figure 5A**), whereas occludin was not affected by SIRT5 silencing (**Figure 5B**).

Discussion

Stroke is an age-related disease and the second-leading cause of death and disability worldwide; furthermore, its incidence is expected to rise due to the continuously increasing life expectancy. To date, the only available treatment is early recanalization which is crucial for improving prognosis in AIS patients.[28] Nonetheless, reperfusion therapy can only be performed within a limited time frame from onset of symptoms and is associated with impaired BBB integrity and increased risks of intracerebral hemorrhage.[6] Loss of BBB integrity correlates with worsened long-term outcome in AIS patients however, the mechanisms underlying this correlation are not clear.[7] In light of the above, it is essential to identify novel molecular targets modulating BBB breakdown after reperfusion so as to develop therapeutic strategies for the treatment of AIS patients.

In the present study, we demonstrate for the first time that genetic deletion or silencing of SIRT5 yields protective effects in the acute phase of ischemic stroke. Indeed, deletion of SIRT5 reduced ischemic brain damage, improved neurological outcome and decreased systemic inflammation through blunted BBB disruption and occludin degradation. *In vivo* findings observed in the mouse were partially reproduced in HBMVECs highlighting the translational relevance of our findings. Accordingly, HBMVECs exposed to H/R after SIRT5 silencing displayed decreased endothelial permeability and increased expression of TJPs occludin and claudin-5 via activation of the PI3K/Akt pathway.

Our findings demonstrate a 50% reduction in stroke size in mice lacking SIRT5 protein; further, we observed a concomitant improvement in neurological deficits at 48 h, but not 24h, after tMCAO underscoring the physiological relevance of the observed effect. Our data are in contrast to previous work describing a protective role of SIRT5 in the heart following ischemia/reperfusion,[13] and thus imply a deleterious role of SIRT5 which is specific for the brain. Similarly to the data obtained in $SIRT5^{-/-}$ mice, *in vivo* knockdown of SIRT5 led to a

33% reduction in stroke size 24 h after tMCAO although, a parallel improvement in neurological deficit could not be confirmed. The latter result was rather unexpected and may be explained by a gene-dosing effect whereby knockdown only yielded a 40% decrease in SIRT5 protein as opposed to SIRT5$^{-/-}$ mice where SIRT5 protein was completely abrogated. Furthermore, the Bederson-based scale used to asses neurological impairment may not be the most suited approach for detecting minor changes despite being a very well established protocol.[29] On the other hand, confirmation of the deleterious role of SIRT5 on stroke size using a knockdown approach adds value to the present study since *in vivo* knockdown by siRNA represents a clinically relevant therapeutical strategy as recently confirmed in several clinical trials.[30, 31] Noteworthy, the use of knockdown animals is also relevant for excluding potential compensatory mechanisms which may take place in knockout animals where the protein of interest is lacking congenitally.[32] Lastly, as previously shown by our group intravenous administration of siRNA primarily targets brain endothelial cells,[18] thereby underscoring the importance of cerebral/endothelial SIRT5 in determining BBB damage and brain injury.

BBB disruption is a hallmark of stroke, however, it is still controversially discussed whether it is the cause or rather the consequence of brain injury. Studies using interventions specific to the endothelium support the former view and indicate the BBB as the cause rather than the consequence of brain damage.[24, 33] In line with this concept, it is of utter importance to study the spatial and temporal changes in BBB breakdown occurring after stroke and the mechanisms responsible for it. Shi et al. observed an early increase in the BBB permeability to small macromolecules (≤3 kDa) at 30 min after ischemia, whereas the increase to larger macromolecules (≥40kDa) such as IgG or serum albumin occurs 3 h after and keeps increasing progressively up to 24 h after ischemia. Extravasation of larger macromolecules across the BBB is associated with upregulation of MMPs and loss of TJPs integrity.[24] In line with this data, we observed IgG extravasation and degradation of claudin-5 and occludin protein 48 h after ischemia. Importantly, SIRT5$^{-/-}$ mice blunted IgG extravasation and occludin

degradation indicating SIRT5 as a novel target against stroke-induced BBB leakiness and TJPs degradation.

The systemic inflammatory response occurring after AIS has been proposed as a prognostic biomarker.[34] Neutrophil count and neutrophil/lymphocyte ratio (NLR) are augmented in the first 3 days after onset of symptoms in stroke patients and correlate with stroke outcome.[35,36] In addition, experimental data showed a positive association between systemic and local inflammatory response after tMCAO. In this regard, an increase in peripheral neutrophil count is associated with enhanced recruitment of leukocyte in cerebral microcirculation and neutrophil activation in the brain.[37] This experimental and clinical evidence supports the notion that systemic inflammatory responses may parallel local inflammatory responses following stroke. Interestingly, our findings reveal a decreased systemic inflammatory response in SIRT5$^{-/-}$ mice as compared to WT mice which maybe the result of attenuated brain injury as observed in SIRT5$^{-/-}$ mice, although the cause-effect relationship remains to be established. Notwithstanding, these data concur with clinical studies in that reduced neutrophil count as well as NLR is associated with improved outcome after stroke.

To assess the translational relevance of our murine findings, we established an *in vitro* model of HBMVECs exposed to H/R. Similarly to our *in vivo* data, SIRT5 silencing attenuated the increase in endothelial permeability following H/R. Moreover, this functional change was paralleled by an upregulation of claudin-5 and occludin protein suggesting this, likewise in vivo, as the responsible mechanism for the reduced permeability following SIRT5 knockdown. Several studies reported that the PI3K/ Akt signaling pathway blunts stroke-induced BBB disruption[38-40] however, the molecular mechanisms underlying this effect are incompletely understood. In line with this observation we coincubated HBMVEC with wortmannin and found that it could abrogate the protective effects achieved by SIRT5 knockdown, thereby providing direct evidence that SIRT5 signals through the PI3K/Akt pathway. In addition, we demonstrate that claudin-5 and occludin coimmunoprecipitate with Akt underscoring a direct interaction. Binding of claudin-5 to Akt was increased following

SIRT5 knockdown suggesting that the claudin-5/Akt interaction may contribute to the upregulation of claudin-5 upon SIRT5 silencing. Claudin-5 and occludin are proteins with relatively short half-life which can be degraded by the ubiquitin–proteasome system or the E3 ubiquitin ligase itch, respectively.[41, 42] Indeed, previous data showed that inhibition of glycogen synthase kinase 3b (GSK3β), a downstream target of Akt, upregulates claudin-5 and occludin by decreasing their turnover.[43] Furthermore, Camire et al. suggested that PI3K modules claudin-5 expression by regulating its degradation.[44] In keeping with this concept, we postulate that as a consequence of knockdown of SIRT5, Akt may reduce TJPs turnover by direct interaction, as it is most likely the case with claudin-5, or through downstream targets such as GSK3β. Nevertheless, additional experiments are warranted in order to confirm the mechanisms underlying SIRT5 siRNA induced Akt-dependent, upregulation of TJPs.

One potential limitation of the present study is that deletion or knockdown of SIRT5 was not limited to brain endothelial cells. Consequently, we cannot exclude potential effects from other organs and/or cell types. On the other hand, our in vitro data confirm the crucial contribution of brain endothelial cells, thereby supporting the proposed mechanism. Another limitation could be related to our *in vitro* model of H/R. TJPs were not downregulated after 4 of hypoxia followed by 4 h of reoxygenation as unlike what we observed *in vivo* following stroke. Nevertheless, other groups did not observe reduced expression of occludin and claudin-5 after hypoxic exposure.[45, 46] Independently of the above, our *in vitro* model showed a decrease in BBB function after exposure to H/R which was attenuated following SIRT5 knockdown.

In summary**,** this study provides first evidence for of SIRT5 in mediating brain injury and neurological deficit in a mouse model of ischemic stroke. This effect was mediated, at least in part, by an increase in BBB permeability and tight junction proteins disruption and was reproduced also in primary HBMVECs. Findings in the present study provide a novel

molecular mechanism involved in BBB degradation following stroke and could provide a concrete platform for the development of novel therapeutic strategies.

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Conflict of Interest

None.

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Figures Legend

Figure 1. Deletion and knockdown of SIRT5 decreases infarct volume after stroke. (A, B) Brain infarct volume at 48 h after tMCAO was measured on TTC-stained coronal sections of WT and SIRT5$^{-/-}$ mice (n= 9). (C) The changes in neurological scores at 2 h, 24 h, and 48 h following tMCAO in WT and SIRT5$^{-/-}$ mice (n=13-12). (D, E) Brain infarct volume at 24 h after tMCAO was measured on TTC-stained coronal sections of scrambled treated mice (siSCR) and SIRT5 treated mice (siSIRT5) (n=10-7). Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 versus WT (B, C); siSCR (E)

Figure 2. Deletion of SIRT5 attenuates BBB disruption and occludin loss after stroke. (A) Representative images showing the extravasation of plasma IgG (green) into the brain parenchyma 48 h after tMCAO. (B) Volume of extravasation of endogenous IgG. (n=6-5) **P*<0.05 versus WT. (C and E) Immunostaining of occludin and claudin 5 in contralateral and ipsilateral hemispheres of WT and SIRT5$^{-/-}$mice. (D and F) Quantification of occludin and claudin-5 positive areas normalized to the total endothelial cell surface area (CD31 staining) in contralateral and ipsilateral hemispheres of WT and SIRT5$^{-/-}$ mice (n=5). Scale bar: 30 µm. Data are expressed as mean ± SEM.**P*<0.05, ***P*<0.01, ****P*<0.001

Figure 3. Deletion of SIRT5 lowers the systemic inflammatory response 48 h after tMCAO. (A, B, C, D) WBC, lymphocyte, neutrophil, counts and neutrophils to lymphocytes ratio in WT and SIRT5$^{-/-}$ mice (n=7-5). Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01 versus WT. WBC= white blood cells.

Figure 4. Knockdown of SIRT5 decreases BBB permeability and upregulates TJPs via PI3K/Akt pathway after exposure to hypoxia and reoxygenation (H/R) in HBMVECs. (A) Western blot analysis showed increase in p-Akt/ Akt ratio after H/R in SIRT5 siRNA compared to scrambled treated cells (n=6). (B) Transendothelial Electrical Resistance (TEER) was measured continuously over 48 h in HBMVECs treated with SIRT5 siRNA and DMSO, scrambled siRNA and DMSO or SIRT5 siRNA and wortmannin (10^{-7} M). Resistance

measurements are normalized to its zero value (baseline period before hypoxia) and plotted as different times after hypoxia (n=10). *P<0.05, ** P<0.01 vs. siSCR # P <0.05 vs. SIRT5+ WM (C, E) Western blot analysis showed upregulation of occludin and claudin-5 in SIRT5 treated cells after 4 h hypoxia followed by 4 h of reoxygenation. Wortmannin (10^{-7} M) pretreatment prevented the upregulation of TJPs induced by SIRT5 knockdown (n=5-10). Data are expressed as mean ± SEM. *P<0.05, ** P<0.01.

Figure 5. Knockdown of SIRT5 enhances the binding of claudin-5 to Akt. (A) Representative immunoblot of coimmunoprecipitation of Akt and claudin-5 from whole-cell lysates of scrambled or SIRT5 siRNA treated cells after H/R. (B) Representative immunoblot of coimmunoprecipitation of Akt and occludin from whole-cell lysates of scrambled or SIRT5 siRNA treated cells after H/R (n = 3)

Figure 1

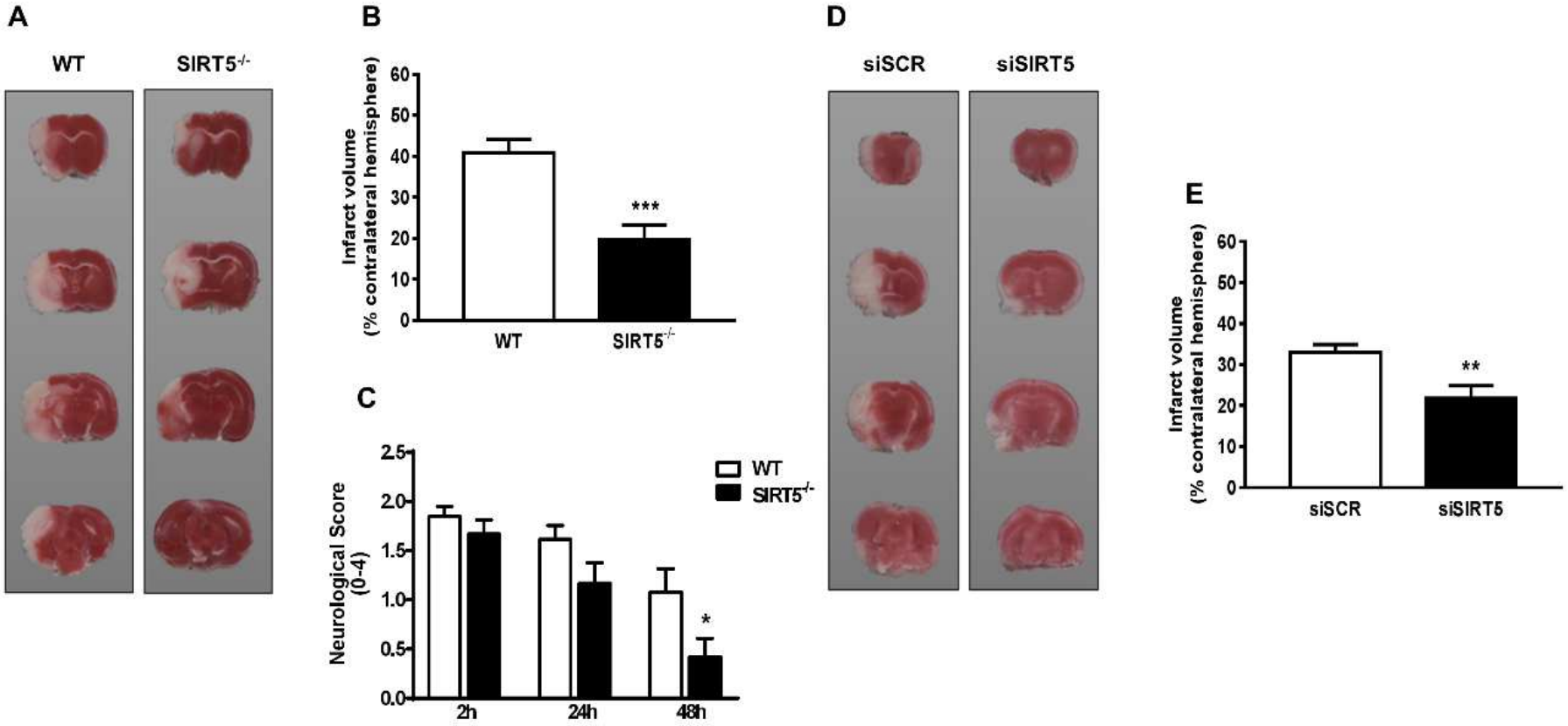

A
WT
SIRT5-/-
B
Infarct volume
(% contralateral hemisphere)
60
50
40
30
20
10
0
***
WT
SIRT5-/-
C
Neurological Score
(0-4)
2.5
2.0
1.5
1.0
0.5
0.0
WT
SIRT5-/-
*
2h
24h
48h
D
siSCR
siSIRT5
E
Infarct volume
(% contralateral hemisphere)
60
50
40
30
20
10
0
**
siSCR
siSIRT5

Figure 2

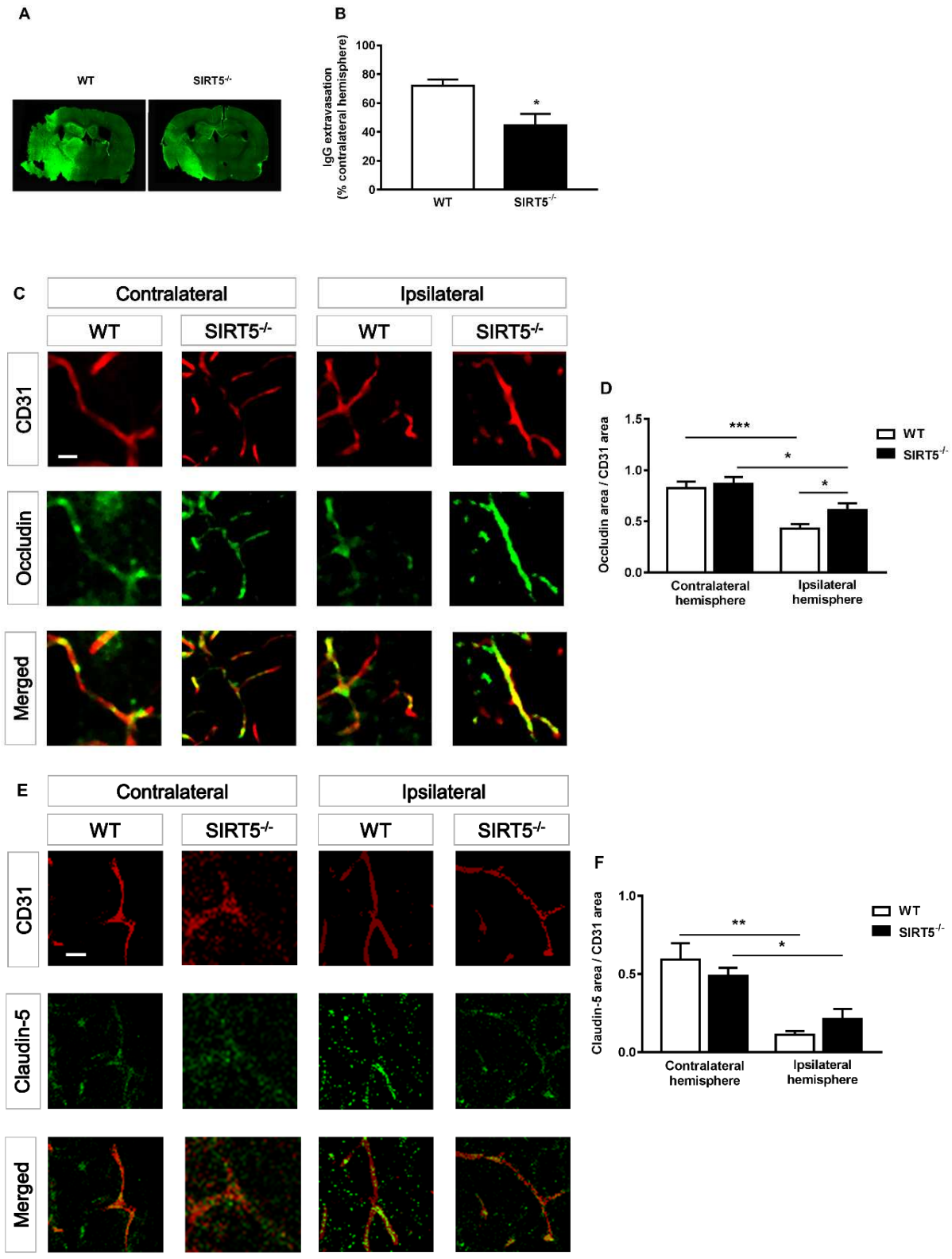
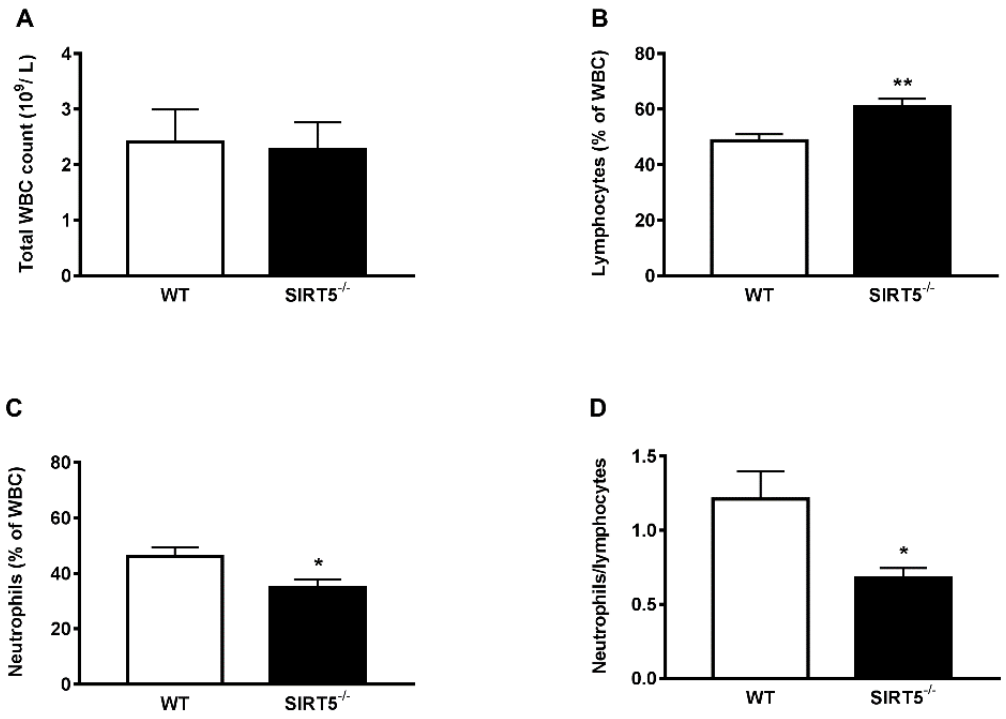
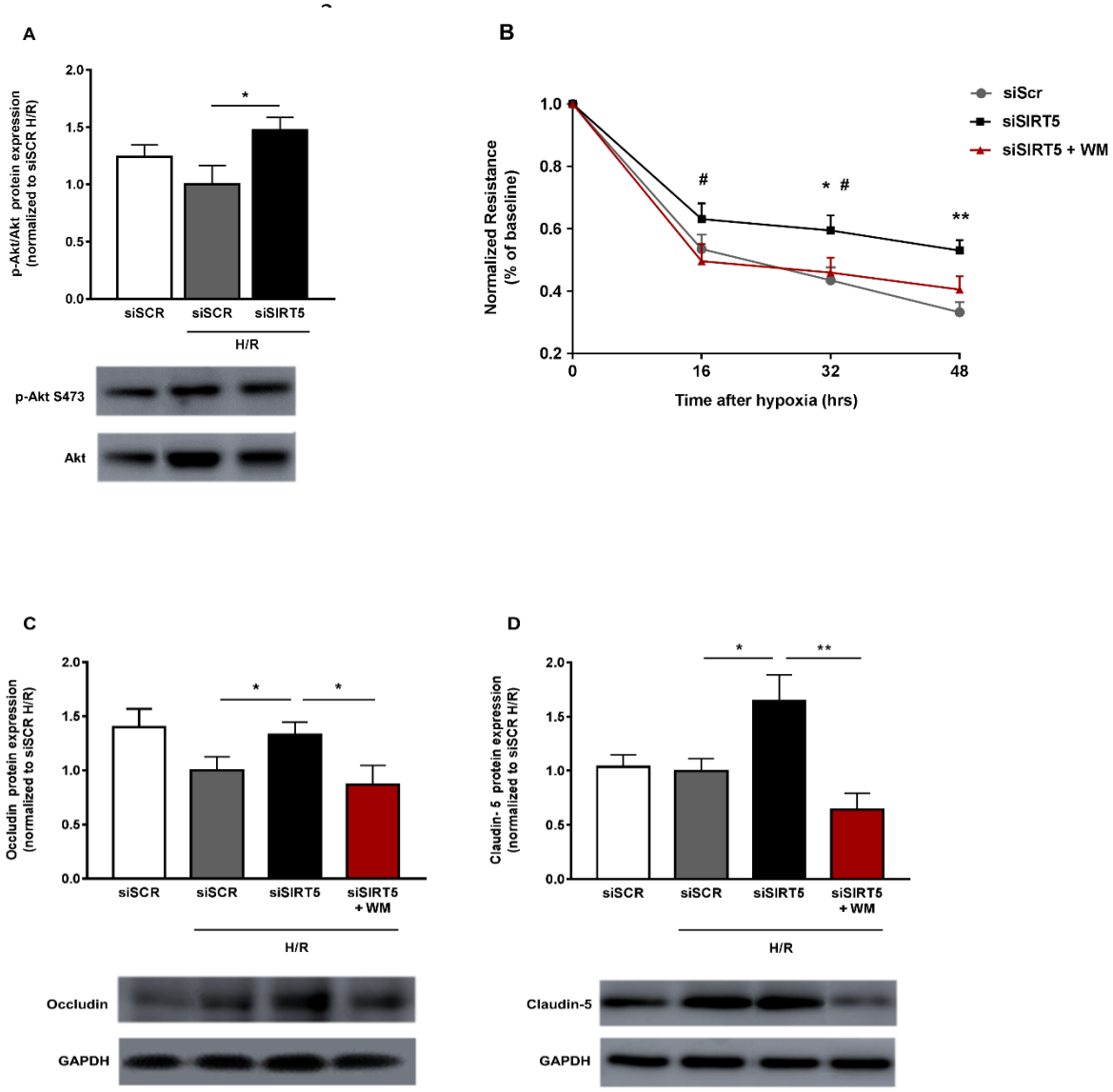

A
WT
SIRT5-/-
B
IgG extravasation (% contralateral hemisphere)
100
80
60
40
20
0
*
WT
SIRT5-/-
C
Contralateral
Ipsilateral
WT
SIRT5-/-
WT
SIRT5-/-
CD31
Occludin
Merged
D
Occludin area / CD31 area
1.5
1.0
0.5
0.0
***
*
*
WT
SIRT5-/-
Contralateral hemisphere
Ipsilateral hemisphere
E
Contralateral
Ipsilateral
WT
SIRT5-/-
WT
SIRT5-/-
CD31
Claudin-5
Merged
F
Claudin-5 area / CD31 area
1.0
0.5
0.0
**
*
WT
SIRT5-/-
Contralateral hemisphere
Ipsilateral hemisphere

Figure 3


A
Total WBC count (10^9/ L)
0
1
2
3
4
WT
SIRT5$^{-/-}$
B
Lymphocytes (% of WBC)
0
20
40
60
80
**
WT
SIRT5$^{-/-}$
C
Neutrophils (% of WBC)
0
20
40
60
80
*
WT
SIRT5$^{-/-}$
D
Neutrophils/lymphocytes
0.0
0.5
1.0
1.5
*
WT
SIRT5$^{-/-}$

1 **Figure 4**


A
p-Akt/Akt protein expression (normalized to siSCR H/R)
2.0
1.5
1.0
0.5
0.0
*
siSCR
siSCR
siSIRT5
H/R
p-Akt S473
Akt
B
Normalized Resistance (% of baseline)
1.0
0.8
0.6
0.4
0.2
#
* #
**
0
16
32
48
Time after hypoxia (hrs)
siScr
siSIRT5
siSIRT5 + WM
C
Occludin protein expression (normalized to siSCR H/R)
2.0
1.5
1.0
0.5
0.0
*
*
siSCR
siSCR
siSIRT5
siSIRT5 + WM
H/R
Occludin
GAPDH
D
Claudin- 5 protein expression (normalized to siSCR H/R)
2.0
1.5
1.0
0.5
0.0
*
**
siSCR
siSCR
siSIRT5
siSIRT5 + WM
H/R
Claudin-5
GAPDH

1 **Figure 5**


A
H/R
siSCR
siSCR
siSIRT5
IB: Claudin-5
IP: AKT
B
H/R
siSCR
siSCR
siSIRT5
IB: Occludin
IP: AKT

2